A mannose receptor-like molecule likely serves as the mate recognition pheromone receptor in the male rotifer *Brachionus manjavacas*

A Thesis Presented to the Academic Faculty by

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Abstract

Mate recognition in the male rotifer *Brachionus manjavacas* is controlled by contact chemoreception, which occurs when the male has a head-on encounter with a conspecific female. The female expresses a glycoprotein, the mate recognition pheromone or MRP, on her body surface that males detect with a receptor located in the male corona. A positive match between signal and receptor causes males to initiate mating behavior. After surveying the female *B. manjavacas* and male *Brachionus plicatilis* transcriptomes for all receptor genes, a C-type lectin gene was found which codes for a mannose receptor (MR). This gene is a good candidate for the MRP receptor because the MRP is glycosylated with mannose and mannose receptors recognize mannose/glucose oligosaccharides. Mannose has been found to block the binding of MR antibodies in males. In addition, mannose receptor immunostains show localized binding at the corona of males, but not in females. Mannose has also been shown to block circling, but binding of antibodies for the human mannose receptor does not decrease circling. A Western blot analysis of female rotifers shows that there is an MR in the female proteome; however, Western blots have not demonstrated binding of MR antibodies to male proteins. Mating bioassays showed that mannose is an important inhibitor of male mate recognition. These findings are best explained by the hypothesis that the male MRP receptor is an MR-like molecule that functions as a C-type lectin that is capable of binding of mannose.

Common abbreviations

ASW	artificial seawater
PBS	phosphate buffered saline
MRP	mate recognition pheromone
MR	mannose receptor
RNAi	RNA interference
dsRNA	double stranded RNA
RE	resting egg
MMP	α-methyl-D-mannopyranoside
MGP	α-methyl-D-glucopyranoside

Introduction

Importance of rotifers

Rotifers, including the species *Brachionus manjavacas*, are a vital part of the food web in marine environments as they link phytoplankton to fish and other predators (Carpenter, Kitchell et al. 1987; Wallace 2002). Since rotifer resting (diapausing) eggs are of potential value for aquaculture, elucidating additional information about sexual reproduction can contribute to increased controlled resting egg production (Rico Martinez and Snell 1997).

Relevance of chemical signaling in rotifers

Due to their use of chemoreception, rotifers are very sensitive to changes in the chemical composition of their environment; in this way, they are useful test organisms for assessing toxin levels (Snell and Janssen 1995; Snell and Joaquim-Justo 2007). The ease of culturing rotifers in the laboratory allows for ecological toxicity testing as well (Snell and Janssen 1995). Changes in rotifer behavior can be used to monitor the ecological stress they are under, for example, by conducting bioassays to evaluate mating behavior.

Chemical signals are also responsible for triggering sexual reproduction (mixis) in monogonont rotifers, including *B. manjavacas*, which are haplodiploid cyclical parthenogens and have retained sexual reproduction despite the ability to proliferate asexually (Wallace 2002; Snell, Kubanek et al. 2006). Sexual reproduction is conserved due to the necessity of the resting egg state, which is the result of a mating event between a male and a mictic female (Fussmann, Ellner et al. 2003). The asexual state, which is characterized by amictic females producing clonal diploid eggs that hatch into amictic females, dominates and only switches to the sexual cycle following chemical cues (Wallace 2002; Kubanek and Snell 2008). These chemical signals are hormones that are released and trigger density-dependent mixis (Kubanek and Snell 2008). Once the threshold density is reached that triggers mixis, the females begin to produce mictic daughters, which in turn produce haploid eggs. If these haploid eggs are fertilized, the egg will give rise to a haploid male. Resting eggs are the result of a mating event between a haploid male and a mictic female. Thus, mictic females can produce haploid eggs or, if fertilized, resting eggs (cysts)

(Wallace 2002). Resting eggs undergo diapause before hatching into amictic females. Hatching of the resting egg is triggered by conditions of increased light, temperature, and moisture, which correspond to the beginning of a new growing season (Gilbert and Schroder 2004). Thus, at least one explanation for the conservation of sexual reproduction in *B. manjavacas* is the necessity of the resting egg to survive harsh environmental conditions.

Relevance of contact chemoreception

Chemical signaling is also responsible for communication between individuals through contact chemoreception (Snell and Morris 1993). The differences in mate recognition among different species of rotifers contribute to the maintenance of reproductive isolation among these species (Rico Martinez and Snell 1997). Mechanisms of reproductive isolation are of interest to evolutionary biologists, as these contribute to speciation.

Rotifer mating behavior

Given the role of chemoreception and hormonal cues in rotifer biology, it follows that mate recognition is also mediated by chemical signaling (Gilbert 1963; Snell and Morris 1993; Snell, Rico Martinez et al. 1995). In *B. manjavacas*, the role of the female in mating is completely passive, whereas the male exhibits a characteristic mating behavior (Gilbert 1963). A male initiates mating when he contacts the female head-on; otherwise no mating behavior is seen. Mating behavior includes the male circling round the female and inseminating by injecting his sperm into the female's body cavity, especially through the corona (Gilbert 1963). Other rotifer species also exhibit this mating behavior (Joanidopoulos and Marwan 1999; Velazquez-Rojas, Santos-Medrano et al. 2002). The mate recognition that leads to this species-specific mating behavior is explained by the action of contact chemoreception when the male encounters the female (Gilbert 1963; Snell and Morris 1993; Snell, Rico Martinez et al. 1995). The female expresses a glycoprotein, the mate recognition pheromone (MRP), on her body surface that serves as the chemical signal that triggers male mating (Snell and Nacionales 1990; Snell and Morris 1993; Snell, Morris et al. 1993; Snell, Rico Martinez et al. 1995).

Nature of the MRP

Snell et al. (1995) report that MRP is a 29 kilodalton protein glycolsylated with mannose, fucose, and N-acetylglucosamine, and is bound to the body surface of female rotifers, especially at her corona, but to a lesser extent at the opening in the lorica at the base of the foot. In correspondence with the highest densities of MRP, most copulations occur in the corona, but 15% take place at the base of the foot (Snell, Rico Martinez et al. 1995). Snell et al. (1995) report that males detect this glycoprotein signal with a receptor located in the male corona, leading to male mating behavior. However, the nature of the male MRP receptor is not well characterized and thus, is the subject of my investigations.

Snell & Nacionales (1990) demonstrated the necessity of the carbohydrate moiety of MRP in mate recognition by conducting experiments where lectins were used to block male recognition of females, thus altering mating behavior. In the same study, female rotifers that had been treated with the glycohydrolase *N*-glycanase, which specifically cleaves *N*-linked oligosaccharides, elicited 65% less male mating behavior than control females (Snell and Nacionales 1990). This indicates that the male recognizes the female based on the presence of the carbohydrate moiety as opposed to the peptide portion of the MRP.

Effect of MRP on mating behavior

Males treated with purified MRP showed circling behavior following a male-female encounter only 5% of the time, compared to control males which initiated circling 65% of the time (Snell, Rico Martinez et al. 1995). In the same study, Snell et al. (1995) showed that denatured MRP did not inhibit mating. Beads coated with MRP were sufficient to elicit mating behavior from male rotifers, indicating that this pheromone is involved in the contact chemoreception that mediates mate recognition (Snell, Rico Martinez et al. 1995). Snell et al. (1995) also determined that the male receptors are concentrated in the corona. There was also a significant reduction in mating attempts by males when females are treated with anti-MRP polyclonal antibody (Snell, Rico Martinez et al. 1995).

MRP receptor localization on males

Males which were exposed to biotinylated MRP and then to fluorescein-labeled beads cross-linked to avidin showed localized binding at the corona, whereas control males showed no visible fluorescence (Snell, Rico Martinez et al. 1995). This indicates that the male MRP receptor is localized at the coronal region.

Role of MRP in reproductive isolation

The interaction between MRP and the MRP receptor may be important for maintaining reproductive isolation among different rotifer populations. Geographically separated groups of rotifers in the Tampa Bay estuary in Florida, USA showed clear mating preferences, which may indicate incipient reproductive isolation (Snell, Rico Martinez et al. 1995). Snell et al. (1995) suggested that changes in the molecular structure of the MRP may be important in the mechanisms of speciation and the evolution of reproductive barriers in rotifers.

Cases of recognition-mediating proteins maintaining reproductive isolation have been reported by other authors, such as Palumbi (1992), who showed that species boundaries between four sympatric sea urchin species in the genus *Echinometra* are maintained by sperm attachment mechanisms. He reported that sperm fail to attach to and penetrate heterospecific eggs, and that attachment is mediated the interaction between a protein called bindin and a glycoprotein receptor on the surface of eggs (Palumbi 1992). Differences in amino acids in bindin among species are associated with the failure of sperm in fertilizing heterospecific eggs (Palumbi 1992). This study is relevant to the study of mate recognition in *B. manjavacas* because binding is analogous to MRP because it is a recognition-mediating protein. Furthermore, amino acid changes present in bindin contribute to reproductive barriers in *Echinometra*; the same could be true for amino acid differences in MRP contributing to reproductive isolation in *B. manjavacas.*

Potential MRP receptors—lectins

Given that MRP is a glycoprotein, a good candidate for an MRP receptor would be a lectin, which is a protein that binds carbohydrates. C-type lectins, which require calcium to bind their sugar

ligand, are a large group of carbohydrate-binding proteins that play a large role in recognition. Mannose receptors (MR) are an important subset of C-type lectins (Stahl and Ezekowitz 1998).

Mannose receptors

The human MR is a 180 kilodalton transmembrane protein with three extracellular binding sites that are capable of recognizing a wide range of endogenous or exogenous ligands, hence they play an important role in immune functions, especially in macrophages and dendritic cells (Gazi and Martinez-Pomares 2009). Structurally, the MR has five domains, each with distinct functions: the amino terminus comprises a cysteine-rich region, followed by a domain containing a fibronectine type II repeat, followed by eight carbohydrate recognition domains (CRDs) which recognize mannose and fucose residues, followed by a transmembrane domain, and finally the carboxy terminal domain located in the cytoplasm (Stahl and Ezekowitz 1998).

C-type lectins recognize their ligands because of the presence of carbohydrate recognition domains (CRDs). These CRDs allow C-type lectins to mediate cellular adhesion as well as pathogen recognition (Cambi, Koopman et al. 2005).

Wootton et al. (2007) have recently found that a mannose-binding C-type lectin mediates prey recognition in the protozoa *Oxyrrhis marina*, a marine dinoflagellate.. This is an example of a C-type lectin mediating recognition in a way that is analogous to how the MRP receptor facilitates mate recognition in *B. manjavacas*.

Objective and hypothesis

The objective of this study is to characterize the male MRP receptor in the male rotifer *Brachionus manjavacas*. Our hypothesis, that the MRP receptor is a C-type lectin, was developed following a series of experiments designed to reveal the binding properties of the putative MRP receptor and a survey of *Brachionus spp.* transcriptomes. Our results support the lectin hypothesis and provide an explanation for how the female signal and male receptor interact to produce mate recognition.

Methods and Materials

Identifying candidates for MRP receptor

The *B. manjavacas* female and *Brachionus plicatilis* male transcriptomes were surveyed (GMOD database) for potential MRP receptors by identifying C-type lectins in the collection of all receptors. Table 1 shows the candidates for MRP receptors.

Table 1: List of potential MRP receptors from *B. manjavacas* female *or B. plicatilis* male transcriptomes; highlighted receptors are candidates for the MRP receptor.

Gene	Animal	Type	Database	Contig	e-value	bp
OR4D6	Human	Olfactory receptor	Bm	2452	0.14	444
Olr325	Rat	Olfactory receptor	Bm	2452	0.57	444
SCAR3	Human	Scavenger receptor A	Bm	3726	4e-4	440
T2R38	Human	Taste receptor	Bm	5721	1.9	525
Taste recept	Drosophila	Gustatory receptor	Bm	5061	1.7	291
OR11-298	Human	Olfactory receptor 8G5	Bm	2395	0.81	323
9Q2	Human	Olfactory receptor	Bm	4758	0.56	444
TonB	Caulobacter	TonB receptor	Bm	3218	9e-24	715
Leptin recept	Mouse	Leptin receptor	Bm	3556	3e-16	542
Notch1	Strong. purpuratus	Receptor protein Notch1	Bm	5755	8e-44	882
Inositol receptor	Human	inositol 1,4,5-triphosphate **receptor**	Bp male	2609	1e-46	512
ACR-21	C.elegans	Acetylcholine receptor family	Bp male	2026	9e-21	748
Tachykinin receptor	Strong. purpuratus	Tachykinin receptor	Bp male	2054	7e-14	696
Vomeronasal 2	Rat	Vomeronasal receptor 2	Bp male	2362	1	757
Ecdysis hormone recept	Strong. purpuratus	Ecdysis triggering hormone receptor	Bp male	2542	1e-22	537
GPCR	Strong. purpuratus	GPCR sea urchin	Bp male	2615	9e-06	684
Ser/thre kinase rec	Chicken	Serine/threonine kinase receptor	Bp male	3835	1e-63	535
RACK	Brare	Activated protein kinase C receptor	Bm	1347	1e-149	447
RACK1	Rat	Guanine nucleotide-binding protein, protein kinase C receptor	Bm	1584	1e-149	1401
CG6033-PA	Drosophila	Receptor kinase	Bm	2513	1e-19	708
GPCR	Canis familiaris	GPCR 107	Bm	4679	2e-07	491
IF2B2	Mouse	Insulin-like growth factor 2	Bm	3840	5e-24	339
Growth hormone	Apis mellifera	Growth hormone i	Bm	5552	4e-49	1244
Grk-2	C. elegans	GPCR kinase family	Bp male	4398	6e-07	762
Chemokine recptor	Zebra fish	Chemokine (C-C- motif) receptor 6a	Bp male	1774	2e-22	775
Mannose recptor	Chicken	Mannose receptor C1	Bp male	1893	4e-05	792

Rotifer culture

All rotifers used in these experiments were from a strain of *Brachionus manjavacas*, formerly known as *B. plicatilis* Russian, which has been continuously maintained in this laboratory since 1983. Rotifers were fed the green alga *Tetraselmis suecica* and cultured at 25°C in 15 ppt artificial seawater (ASW).

RNA interference

Double-stranded RNA (dsRNA) of an MR gene from the female *B. manjavaas* was synthesized following the protocol outlined in Snell et al. (2009). RNA interference (RNAi) was performed to attempt to knock down the expression of MR mRNA in the F2 male progeny of treated females. The double transfection protocol for rotifers was developed by Snell et al. (in press) and was followed to transfect resting eggs and the subsequent hatchlings with the dsRNA. Mating bioassays were then performed to test whether there was a phenotypic effect on mate recognition due to the putative RNAi knock down of expression of the MR gene in the male progeny of transfected females. As a control, mating bioassays were also performed using males from untransfected mothers.

RNA interference—preparation of dsRNA

Rotifer genomic DNA was amplified using polymerase chain reaction (PCR) and then subjected to T7 PCR amplification. Attempts to amplify the putative male MR gene were not successful, so the female MR candidate was amplified. dsRNA was prepared from the T7 PCR products using *in vitro* transcription . The dsRNA was used in resting egg and feeding transfections to knock down the expression of the MR gene.

RNA interference—resting egg decapsulation and double transfection

Twenty-five milligrams of resting eggs were hydrated for 15 minutes in 200 µL of 15 ppt ASW in a 2 mL microcentrifuge tube. To this, 600 µL of cold buffered ASW (pH 10) was added followed by 500 µL of fresh bleach. The tube was then vortexed for 10 seconds, incubated for one minute, vortexed for another 10 seconds, and finally incubated for another minute. The contents, including the floating embryos, were poured through a small 44 um filter, which had been soaked in a beaker of sterile water

with a small amount of bleach. The filter was rinsed with ~200 mL of ASW until the chlorine smell subsided. The filter was then filled with ASW containing 0.1% sodium thiosulfate, incubated for one minute, then drained. The embryos were washed from the filter with 5 mL ASW into a 6-well plate. The decapsulated resting eggs were then incubated for 24 hours with the appropriate transfection solution at 25°C in light to hatch.

For the resting egg transfection, about 200 decapsulated resting eggs were placed in 1 mL 15 ppt ASW in a 12-well plate. In a1.5 mL microcentrifuge tube, 20 μL PBS was combined with 5 μL appropriate dsRNA, of 5 μL PBS for the negative control. In a separate 1.5 mL microcentrifuge tube, 2 μL Fugene HD reagent (Roche) was added to 23 μL PBS. The contents of the two tubes was combined and thoroughly mixed by pipeting up and down several times. This transfection mixture was incubated at room temperature for 15 minutes to allow the formation of the transfection complexes, and then the entire 50 μL was added to each well containing 200 decapsulated resting eggs. The plate was incubated for 24 hours at 25°C in light to promote hatching.

For the feeding transfection, about 60 hatchlings per treatment were collected and placed in 0.5 mL ASW in a 24-well plate. The same transfection mixture that was used for the RE transfection was prepared. Following the 15-minute incubation, the entire 50 μL transfection mixture was added to each well. The plate was shaken to mix and incubated at 25°C for 24 hours.

The dsRNA used in both transfections matched the RNA transcript of the gene that codes for the mannose receptor. This double transfection protocol is outlined in Snell et al. (in press).

RNA interference—assessment

The goal of RNA interference (RNAi) is to knock down the expression the MR gene in female rotifers and potentially knock down the expression of MR in the F2 male progeny of the treated females.

After the resting eggs of the species *B. manjavacas* were decapsulated and subjected to the two transfection protocols outlined above, the F2 male progeny of treated females were subjected to a mating bioassay with untreated females.

Mannose Receptor antibody blocking in males

The goal of this experiment is to block male mate recognition by binding MR antibodies to the male MRP receptor, thus blocking the binding of MRP to the receptor.

One hundred males from a filtered culture of rotifers were collected in minimal volume and placed in 1.7 mL microcentrifuge tubes. To the tubes, 100 μL PBS with 1% bovine serum albumin (BSA) was added and the tubes were incubated for 30 minutes to block nonspecific binding. Following the incubation period, 200 μL PBS with 1% BSA containing 1 μg/mL (20 μL of 400 μg/mL stock) of the primary antibody (Rabbit polyclonal antibody to human MR (abcam)) was added to the tube. The tube was incubated for 100 minutes in the dark and then the animals were used in a mating bioassay with untreated females. Two controls were performed; for the first control the males were subjected to a secondary antibody (1 mg/mL stock; pAb to Rb IgG (AP; abcam)), which would be nonreactive with the male rotifers. The negative control involved treating the males with PBS.

In situ hybridization to mannose receptor probe

A fluorescein-labeled dsRNA probe for mannose receptor mRNA was used to identify sites within the animal where MR mRNA synthesis is occurring. The patterns of mRNA synthesis were compared between males and females. As a control, an unlabeled probe for an inositol 1,4,5-triphosphate receptor gene was also performed before exposure to the MR probe to test for non-specific binding.

Immunostaining with a polyclonal mannose receptor antibody

A commercially available polyclonal antibody raised against a human mannose receptor protein was tested for binding to male and female rotifers. Binding was visualized using an FITC-labeled secondary antibody to rabbit immunoglobulin. As a control, binding of a secondary antibody along to male rotifers was tested to determine whether nonspecific binding occured. Additionally, the ability of α-methyl-D-mannopyranoside (MMP) to competitively inhibit antibody binding was assessed using epifluorescence microscopy. The ability of MMP to compete with the male reception of the MRP signal

was evidenced by a reduction in mating attempts in mating bioassays. The controls for these mating bioassays were ASW treated males and males treated with α-methyl-D-glucopyranoside (MGP).

Mannose labeling via avidin-biotin linkage

A 50 mM biotin hydrazide solution was prepared by dissolving 8.8 mg of biotin hydrazide (Thermo 21360) in 350 µL dimethylsulfoxide (DMSO). A 12 mM solution of mannose was prepared by dissolving 1.30 mg mannose in 500 µL deionized water. An oxidation buffer (0.1 M sodium acetate buffer, pH 5.5) was prepared by adding 0.82 g sodium acetate to 100 mL deionized water and then titrated to pH 5.5 with concentrated HCl. For each condition, a 15 mM solution of $NaIO_4$ was prepared by adding 1.7 mg $NaIO_4$ to 500 µL oxidation buffer. The coupling buffer consisted of PBS at pH 7.2.

For the biotinylation, 500 µL mannose solution was combined with 500 µL $NaIO_4$ solution in 1.7 mL microcentrifuge tubes. The tubes were covered and incubated on ice for 30 minutes to oxidize mannose to aldehydes. 250 µL of oxidized mannose was transferred to a new microcentrifuge tube and 650 µL coupling buffer was added along with 100 µL 50 mM biotin solution. These were mixed for two hours at room temperature. A solution of neutravidin-labeled fluorophores was prepared by adding 1.5 µL stock solution to 499 µL deionized water and then vortexing the tube.

For the rotifer labeling and visualization, males and females were isolated from a five-day old culture and washed in ASW. Approximately 50 rotifers of each sex were transferred in 250 µL to a microcentrifuge tube. 250 µL carbonated water was added to anesthetize the rotifers, followed by 5 µL 20% formalin to fix them. Approximately 50 males and females were transferred into new separate microcentrifuge tubes in minimal volume. 50 µL of biotinylated mannose solution was added and mixed for 30 minutes, after which, 30 µL neutravidin bead solution was added and the tube was mixed for an additional 10 minutes. The rotifers were transferred to 600 µL ASW in a 24-well plate. Ten representative males and females were transferred onto a slide and examined using epifluorescence microscopy.

Mating bioassays

Mating bioassays were performed in accordance with the protocol outlined in Snell et al (2009). All animals were collected using narrow bore glass pipettes under 10x magnification. The females used in the mating bioassays were filtered using a 44 um filter from a culture of rotifers in ASW containing *T. suecica.* For each mating bioassay, seven males were placed on a microscope slide with one untreated female. The mating behavior of the animals was observed on a video monitor using 10x magnification for three minutes. During this time, each time a male swam into ("encountered") a female and each time this encounter led to a circling (mating) event was recorded. The ratio of circlings to encounters was determined and this percent circling was used as a measure of mating behavior.

Western Blot

For the SDS-PAGE of rotifer protein, the appropriate quantity of animals (100 female rotifers or approximately 800-1000 male rotifers) was collected in 100 µL ASW in a 1.7 mL microcentrifuge tube and then homogenized using a disposable pestle. For the collection of male rotifers, the animals were collected in multiple tubes, which were then combined after alternating refrigeration and centrifugation to bring the animals to the bottom of the microcentrifuge tubes. To the homogenate, 100 µL 2X sample buffer was added. The 2X sample buffer was prepared by combining 4.0 mL 10% w/v sodium dodecyl sulfate (SDS), 2.0 mL glycerol, 1.0 mL 0.1% Bromophenol Blue, 2.5 mL 0.5 M Tris-HCl (pH 6.8), and 200 µL 2-mercaptoethanol, then brought to 10 mL using deionized water. The tube containing homogenized rotifers and the sample buffer was then heated to 100°C for 5 minutes. The samples were then loaded onto an 8-16% polyacrylamide gel in the amounts of 10-25 µL, at five µL increments, and were run at 130 mA (~80 V) for one hour and thirty to forty-five minutes. The gel was carefully removed and its dimensions were measured. The PVDF membrane was cut to the gel dimensions and the cassette was then assembled. The cassette was assembled by first laying down a fiber pad, then a sheet of filter paper, followed by the gel, followed by the PVDF membrane, then another filter paper sheet, and finally the last fiber pad. The cassette was closed and locked and placed in the box along with a cooling unit and a stir bar. The unit was filled to the top with blotting buffer (25 mM Tris and 192 mM glycine at pH 8.3)

and was run at 350 mA (~100 V) for one hour. The cassette was then removed and the membrane was cut in half at the appropriate lane; one half of the membrane was subjected to a Colloidal Gold total protein stain and the other was subjected to an antibody staining protocol.

Colloidal Gold Total Protein Stain

One half of the membrane was placed in a clean, small glass Petri dish and washed three times for fifteen minutes each with TTBS buffer. The membrane was then washed three times for two minutes each in deionized water. Following these washes, the membrane was covered in approximately 6 mL Colloidal Gold total protein stain and was incubated with gentle agitation for two to three hours to visualize proteins.

Antibody Stain

The other half of the membrane was placed in a small glass Petri dish containing PBS with 1% BSA for thirty minutes to block nonspecific binding. This solution was poured off and the membrane was the soaked in 6 mL PBS with 1% BSA containing 6 µL of MR antibody (700 ug/mL stock used at a concentration of 1 ug/mL; Rb pAb to MR (abcam)). The membrane was incubated for one hour with gentle agitation in this solution. The solution was then poured off and the membrane was briefly rinsed in PBS with 1% BSA. Next, the membrane was incubated in 6 mL of PBS with 1% BSA containing 12 µL of the secondary antibody (1 mg/mL stock diluted 1:500; pAb to Rb IgG (AP; abcam)) for one hour with gentle agitation. The solution was then poured off and the membrane was rinsed twice, each time for 15 minutes, in PBS with 1% BSA. The excess water was allowed to drip off of the membrane, which was then placed in a new small glass Petri dish. The membrane was then subjected to 2.5 mL CDP-Star chemiluminescent reagent for five minutes with shaking. Following this, the excess liquid was again allowed to drip off and the membrane was placed protein side up on a sheet of filter paper and wrapped in Saran wrap. The wrapped membrane was used to expose Kodak BioMax Light film.

To visualize the bands that indicate the presence of a MR antibody-binding protein on the membrane, the wrapped membrane was placed in a light-tight film cassette for fifteen to thirty minutes to expose the film. The film was then developed and analyzed.

Results

Identifying candidates for MRP receptor

Upon surveying the *B. manjavacas* female and *Brachionus plicatilis* male transcriptomes for potential receptor genes, a gene was found that encodes a mannose receptor-like molecule in *B. manjavacas* (pers. comm. Terry Snell; see Table 1). This is a good candidate for the MRP receptor because a C-type lectin MR recognizes mannose and glucose residues and the MRP is glycosylated with mannose (Snell, Rico Martinez et al. 1995).

In situ hybridization to MR probe

Abundant mRNA was observed in the coronal region for the MR probe, as shown in Figure 1 (pers. comm. Eric Tvedte). More intense fluorescence in the photographs corresponds to more binding of probe to MR mRNA.

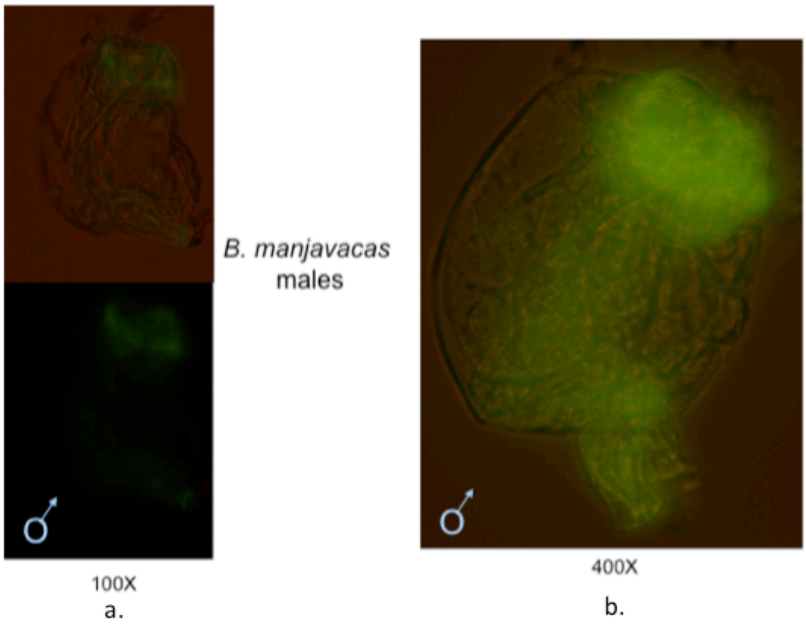

Figure 1: *In situ* hybridization of the MR probes to mRNA in male *B. manjavacas* at 100x magnification (a) and 400x magnification (b).

Immunostaining with a polyclonal MR antibody

The immunostaining assay showed that there was no nonspecific binding of secondary antibody to male rotifers (pers. comm. Eric Tvedte; Figure 2a and 2b). There was binding of MR antibodies observed to occur at the coronal region of males, but binding was not observed in female rotifers (Figure 2c). It was observed that mannose, in the form of α-methyl-D-mannopyranoside (MMP) at a concentration of 500 mM, inhibited the binding of MR antibodies, as evidenced by a decrease in pixel intensity ($p=0.005$; Figure 3). Treatment with MMP decreased mating behavior by males, as shown in a reduction in male percent circling (Figure 4). The mean percent circling for negative control (PBS) treated males was 37.0 ± 5.0 percent circling and was 29.6 ± 4.2 percent circling for the males treated with α-methyl-D-glucopyrannoside (MGP). The mean for the MMP treated males was 16.1 ± 2.9 percent circling, which yields a P-value of 0.0026 from a Fisher's Exact Test when compared to the ASW treated males. The P-value for the comparison of MMP treated males to MGP treated males was 0.034 and the P-value for the comparison of ASW treated males to MGP treated males was 0.222.

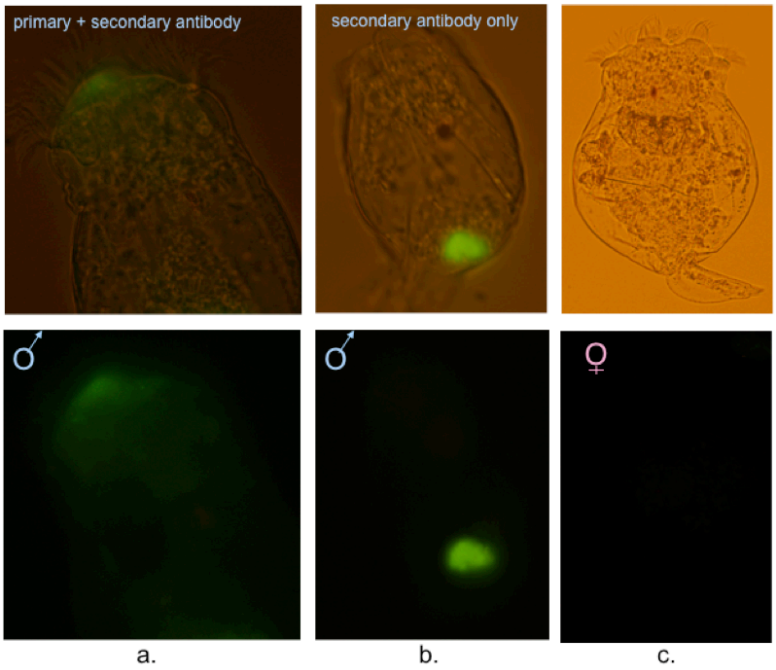

Figure 2: Specific antibody binding in the MR antibody immunostaining. Notice that the coronal region is showing fluorescence where the male was treated with both primary (MR) and secondary antibodies

(a); only autofluorescence is seen in the males treated with secondary antibody alone (b); treating females with MR antibodies resulted in no visible fluorescence, indicating no binding of MR antibodies (c).

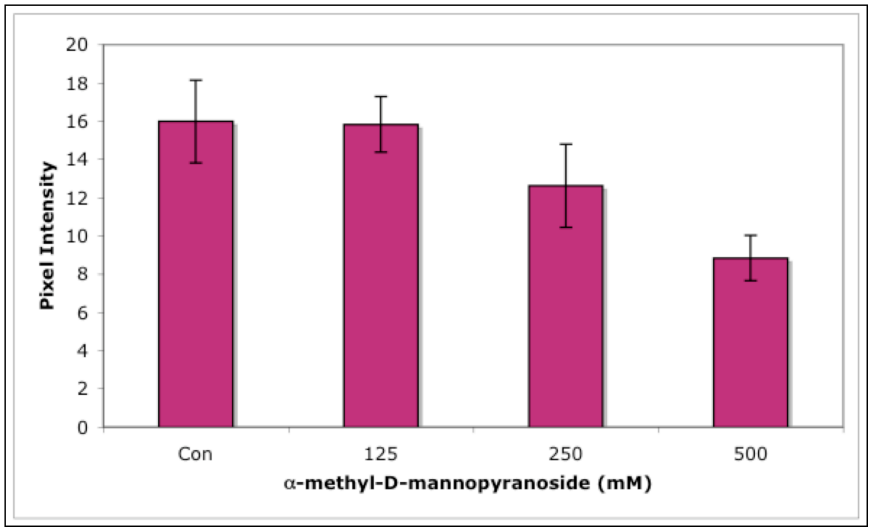

Figure 3: MR antibody binding with varying concentrations of α-methyl-D-mannopyranoside. Mannose blocks the binding of MR antibodies, but only affects fluorescence at certain concentrations, as calculated as a reduction in pixel intensity with increased mannose. P=0.005 for the comparison of control to 500 mM mannose (ANOVA).

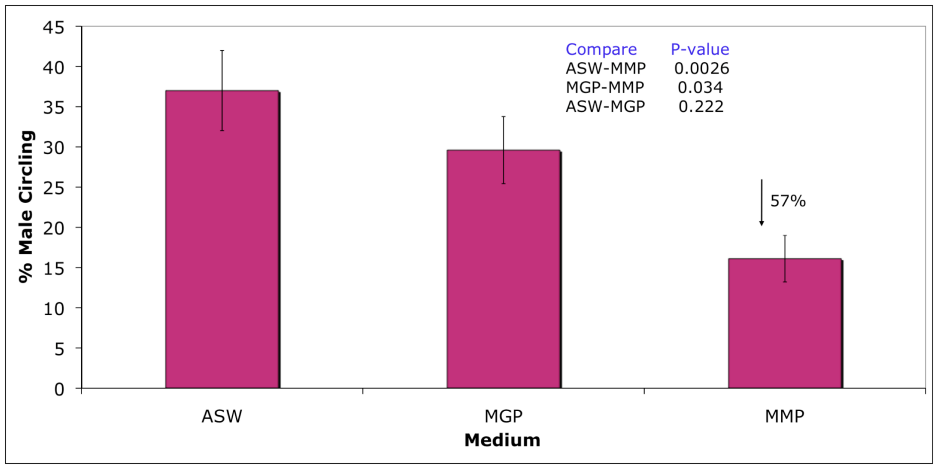

Figure 4: Reduction in mating, measured as percent male circling, obtained by treating males with MMP. The controls are ASW and α-methyl-D-glucopyrannoside (MGP). Significant levels were determined using ANOVA.

Binding of biotinylated mannose to males

The biotinylated mannose binding assays showed significant binding of mannose to the coronal region of males (pers. comm. Daniel Hicks; Figures 5 & 6). The control animals exhibited only autofluorescence, as seen in Figure 6. Males showed greater fluorescence than females (Figure 7), and greater fluorescence intensity was observed in 24-hour-old males, compared to 2-hour-old males (Figure 8). The control and galactose treated males showed only autofluorescence.

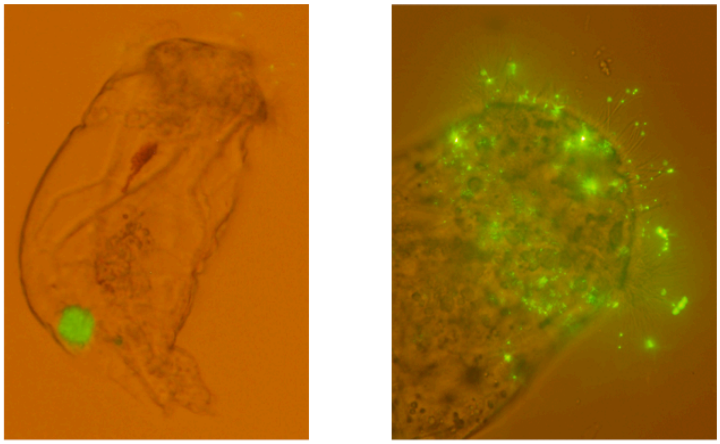

Figure 5: Biotinylated mannose binds to the coronal region of males. The control male on the left exhibits characteristic autofluorescence.

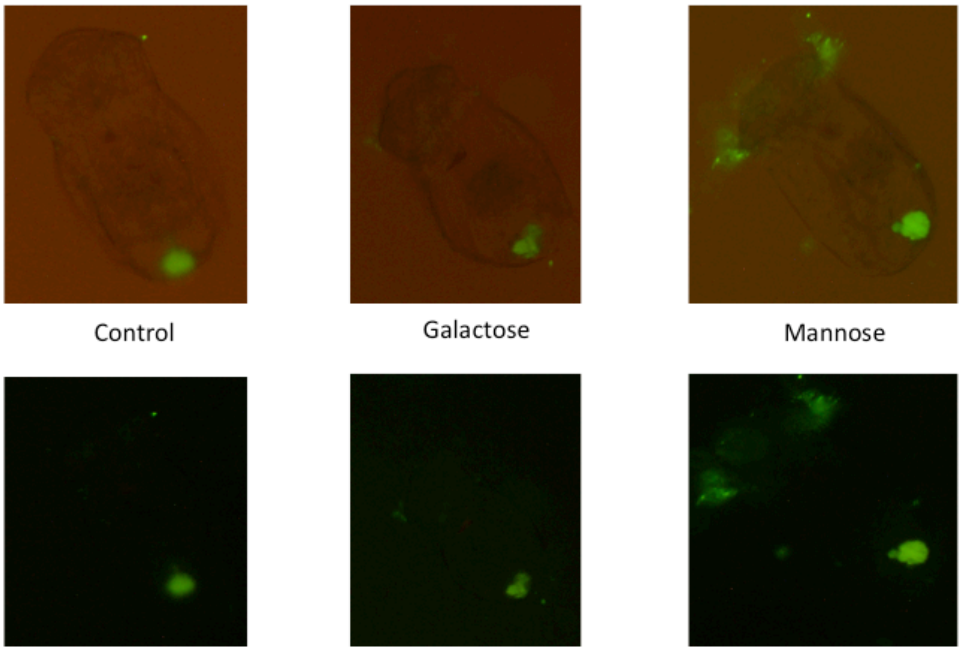

Figure 6: Binding patterns of biotinylated mannose treated males to the negative control and biotinylated galactose treated males. The mannose treatment is the only treatment that shows coronal fluorescence.

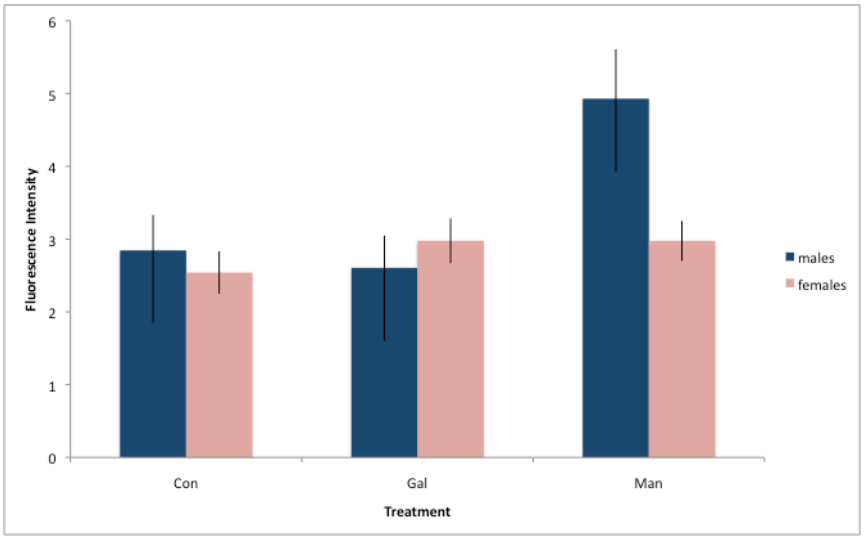

Figure 7: Comparison of the fluorescence intensity between males and females for mannose, galactose, and negative control treatments.

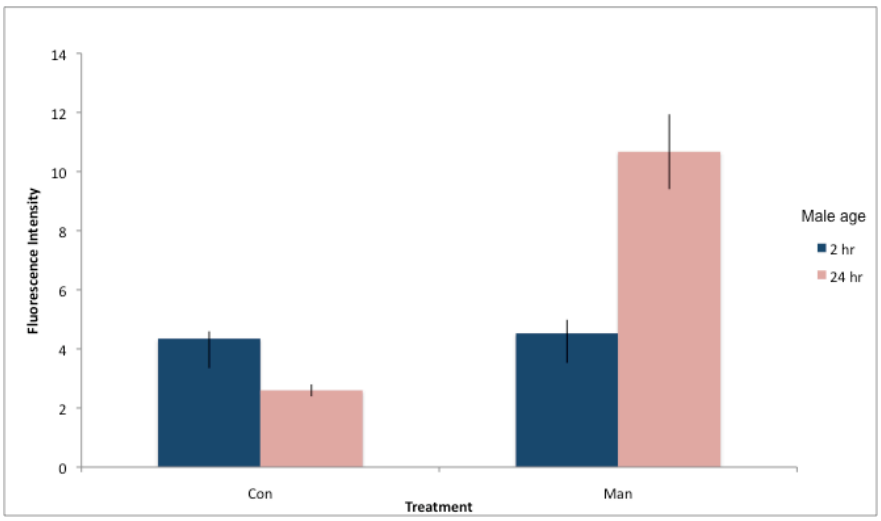

Figure 8: This graph shows the higher fluorescence intensity seen in males treated with biotinylated mannose at the age of 24 hours compared to 2-hour-old males.

Western Blot—Colloidal Gold Total Protein Stain and Antibody Stain

The Colloidal Gold stained membrane was used to visualize the complete rotifer proteome and this was used in conjunction with the antibody-stained membrane to determine the size of any bands that could be seen on the film. The results of the female Western blot showed a clear band at 140 kD corresponding to MR (Figure 9). The male Western blot and staining assays did not show a band corresponding to MR (Figure 10).

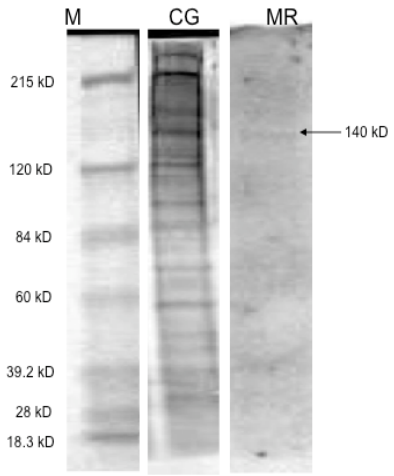

Figure 9: The location of the band on the film that corresponds to the mannose receptor in the female proteome. The lane on the left bears the protein markers with the specified sizes. The middle lane was stained with Colloidal Gold Total Protein Stain and thus stained all of the proteins in the proteome. The lane on the right was stained using mannose receptor antibodies.

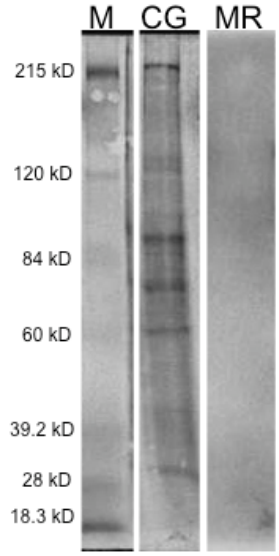

Figure 10: There is no band on the film that binds the mannose receptor antibodies in the male proteome. The lane on the left bears the protein markers with the specified sizes. The middle lane was stained with Colloidal Gold Total Protein Stain and thus stained all of the proteins in the male proteome. The lane on the right was stained using mannose receptor antibodies.

MR antibody blocking in males

The results of the bioassay conducted following the MR antibody blocking in males demonstrated no significant reduction in the percent circling in males that had been treated with the MR antibody compared the untreated males. The percent circling for MR antibody treated males was 37.5±13.8 percent circling. The percent circling for the males treated with secondary antibody was 36.7±5.4 percent circling. The percent circling for the PBS treatment was 38.2±6.2 percent circling.

RNA interference

The results of the RNA interference experiment showed no significant reduction ($p>0.05$) in the percent circlings in male progeny of transfected females compared to untreated males. The percent circling in the male progeny of PBS (control) transfected maternal females was 39.6±11.8 percent circling, compared to the progeny of the MR dsRNA-transfected females at 42.9±9.9 percent circling.

Discussion

Given that the female MRP is a glycoprotein bearing mannose residues, a lectin-like receptor is a good candidate for the MRP receptor on males. When the male transcriptome was surveyed, several candidate receptors were found, including an MR-like C-type lectin, which was considered a putative MR gene from then on. The male MRP receptor was shown to readily bind mannose, as demonstrated by the "Immunostaining with polyclonal MR antibody" experiment, but may not readily bind the human MR ligand, as evidenced by the lack of a reduction in mating attempts by males treated with MR antibodies and used in mating bioassays. This could be attributed to structural differences between the human MR, which the primary antibody was raised against, and the male MRP receptor. The male MRP receptor is therefore concluded to be an MR-like molecule that is structurally distinct from the human MR based on the results of these experiments.

The findings are further supported by the results of the biotinylated mannose binding assays: there was substantially more binding seen in the males treated with biotinylated mannose compared to untreated males or males treated with biotinylated galactose, and when compared to females (see Fig. 7). The fact that 24-hour-old males showed increased binding compared to 2-hour-old males can be explained by the fact that these receptors increase in expression with age as males mature. Additionally, the treatment of males with mannose in the form of α-methyl-D-mannopyranoside significantly decreased mating attempts, indicating that the mannose successfully blocked the MRP receptor from recognizing the female MRP (see Fig.4).

The results of the Western blot assays support the conclusion that the male MRP receptor is structurally distinct from the human MR against which the MR antibodies used in the antibody stain were raised. The female Western blot indicated the presence of a MR capable of binding antibodies to the human MR, but this protein was not detected in males based on the absence of MR antibody binding to male proteins in the Western blot. This may indicate that the female proteome has a protein not found in the male proteome, which is capable of binding the human MR antibodies; this protein may mediate recognition in a wide variety of cells throughout the female rotifer.

The results of the "*In situ* hybridization with an MR probe" experiment indicated that there was MR mRNA being transcribed in the coronal region of male rotifers, which could be explained by possible post-translational modifications of the male MR, based on the fact that the male Western blot did not detect the presence of an MR in the proteome. Furthermore, while MR antibodies were found to bind to the male corona in the immunostain assays, there was a greater affinity for mannose than for MR antibodies at these sites, as the addition of α-methyl-D-mannopyranoside decreased the intensity of MR antibody binding. This supports the idea that the receptor is similar to an MR but structurally distinct, ensuring that the receptor preferentially binds mannose.

Though the method of gene silencing employed in these experiments, RNA interference, has shown promising results in many organisms, including rotifers (Fire, Xu et al. 1998; Shearer and Snell 2007), there was a lack of significant reduction in mating in the F2 male progeny of female rotifers that had been doubly transfected with MR dsRNA. This may be attributable to the fact that the effects of RNAi did not persist through the F2 generation. The inheritance of RNAi in *Caenorhabditis elegans* has been studied and it was found that wild type gene activity resumes in the F2 progeny of treated maternal females (Montgomery, Xu et al. 1998).

Alternatively, it is possible that the female MR gene that was used in the RNAi experiments had no effect in males. This would imply that even if the RNAi knockdown worked, no effects on mating would be observed since it is unlikely to be knocking down the putative male MRP receptor. This might also explain why the female Western blot achieved a signal but the male Western blot did not show such a signal.

The data presented are explained by the fact that the male MRP receptor may be an MR-like molecule that bears specific structural properties capable of binding mannose but is structurally distinct from the human MR. These data are of value to biologists as the male MRP receptor is critical to mate recognition, and this study provides input on the properties of the receptor. The study of mate recognition and preference in rotifers is important as a model to study how reproductive barriers evolve and are maintained among geographically separated populations, as well as to the analysis of phenotypically

similar groups of rotifers that show mate preference. Furthermore, the male MRP receptor may be a novel C-type lectin as it preferentially binds mannose over glucose.

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